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Titin is a candidate gene for stroke volume response to endurance training: the HERITAGE Family Study

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Rankinen, Tuomo, Treva Rice, Anik Boudreau, Arthur S. Leon, James S. Skinner, Jack H. Wilmore, D. C. Rao, and Claude Bouchard. Titin is a candidate gene for stroke volume response to endurance training: the HERITAGE Family Study. *Physiol Genomics* 15: 27–33, 2003. First published July 15, 2003; 10.1152/physiolgenomics.00147.2002.—A genome-wide linkage scan for endurance training-induced changes in submaximal exercise stroke volume (ΔSV50) in the HERITAGE Family Study revealed two chromosomal regions (2q31-q32 and 10p11.2) with at least suggestive evidence of linkage among white families. Here we report a further characterization of the quantitative trait locus (QTL) in chromosome 2q31 and provide evidence that titin (*TTN*) is likely a candidate gene involved. The original linkage was detected with two markers (*D2S335* and *D2S1391*), and the QTL covered ~25 million base pairs (Mb). We added 12 microsatellite markers resulting in an average marker density of one marker per 2.3 Mb. The evidence of linkage increased from $P = 0.006$ to $P = 0.0002$ and 0.00002 in the multi- and single-point analyses, respectively. The strongest evidence of linkage was seen with two markers in and near the *TTN* gene. Transmission/disequilibrium test (TDT) with the same marker set provided evidence for association with one of the *TTN* markers (*D2S385*; $P = 0.004$). *TTN* is a major contributor to the elasticity of cardiomyocytes and a key regulator of the Frank-Starling mechanism. Since *TTN* is the largest gene in the human genome, the challenge is to identify the DNA sequence variants contributing to the interindividual differences in cardiac adaptation to endurance training.

genetics; fine mapping; exercise training; cardiac function; linkage; association studies

REGULAR ENDURANCE TRAINING induces several beneficial changes in cardiac function, such as reduced heart rate and increased stroke volume at rest and during submaximal exercise at fixed power outputs. These changes display marked interindividual differences, with some subjects showing major changes, whereas others do not respond despite identical training volume and intensity (6, 31). This variability is partly due to genetic factors. In the HERITAGE Family Study, we have reported maximal heritabilities of 41% and 29% for submaximal exercise (50 W) stroke volume (SV50) in the sedentary state and its response to training, respectively (3).

To identify the genes affecting training-induced changes in SV50, we performed a genome-wide linkage scan using 509 polymorphic markers (21). The scan revealed two chromosomal regions (2q31-q32 and 10p11.2) with at least suggestive evidence of linkage with the changes in stroke volume at 50 W (ΔSV50) among white families. We reviewed the chromosome 2q31-q32 linkage area in detail. The region harbors several potential candidate genes, such as nicotinic cholinergic receptor α1 (*CHRNA1*) and titin (*TTN*). We report here that a denser microsatellite mapping increased the linkage signal and that *TTN* appears to be the best candidate gene for human variation in ΔSV50 within the quantitative trait locus (QTL) uncovered in the white HERITAGE families.

METHODS

Subjects. The study cohort consists of 483 white subjects (233 men and 250 women) from 99 nuclear families and 259 black subjects (88 men and 171 women) from 105 family units. Complete training response data were available for 450 whites (216 men, 234 women) and 251 blacks (88 men and 163 women). The maximum number of sib-pairs available was 328 and 102 in whites and blacks, respectively. The study design and inclusion criteria have been described previously (5). To be eligible, the individuals were required to be in good health, i.e., free of diabetes, cardiovascular diseases, or other chronic diseases that would prevent their participation in an exercise-training program. Subjects were also required to be sedentary, defined as not having engaged in regular physical activity over the previous 6 mo. Individuals with a resting systolic blood pressure greater than 159 mmHg and/or a diastolic blood pressure more than 99 mmHg were excluded. The study protocol had been approved by each of the Institutional Review Boards of the HERITAGE Family Study research consortium. Written informed consent was obtained from each participant.

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Submaximal exercise cardiac output and stroke volume. Before and after the 20-wk training program, each subject completed three cycle ergometer (Ergo-Metrics 800S; SensorMedics, Yorba Linda, CA) exercise tests conducted on separate days: a maximal exercise test (Max), a submaximal exercise test (Submax) and a submaximal/maximal exercise test (Submax/Max) (26). The Submax test was performed at 50 W and at 60% of the initial maximal oxygen consumption ($\dot{V}O_{2\,max}$), and subjects exercised for 8–12 min at each work rate, with a 4-min period of seated rest between exercise periods. The Submax/Max test was started with the Submax protocol. After exercising at 60% $\dot{V}O_{2\,max}$, subjects also exercised for 3 min at 80% $\dot{V}O_{2\,max}$. The test then progressed to a maximal level of exertion. Heart rate (HR) and cardiac output (Q) were determined twice at 50 W (HR50 and Q50, respectively). The values presented in this paper represent the mean of the responses for the two submaximal tests (i.e., four individual measurements), both before and after training. Q50 was determined using the Collier CO_2 rebreathing technique (9), as previously described (30). SV50 was derived by dividing Q50 by HR50 (measured with ECG) at the time of the Q50 determination (i.e., SV50 = Q50/HR50). Q50 and SV50 training responses (Δ) were calculated as posttraining values minus pretraining values. The HERITAGE Family Study data collection protocol included a detailed quality assurance and quality control procedures as described elsewhere (12). The reproducibility of the SV50, Q50, and HR50 measurements was good, the coefficients of variation being 7.6, 5.1, and 7.2, and intraclass correlation coefficients being 0.87, 0.89, and 0.76, respectively (32).

Data adjustment. Baseline SV50 and Q50 were adjusted for the effects of sex, age, and body surface area (BSA) using stepwise multiple regression (23). Baseline HR50 was adjusted for sex, age, and body mass index (BMI). Training response phenotypes were adjusted also for baseline value of the phenotype. In summary, SV50, Q50, and HR50 phenotypes were regressed on baseline BSA (HR50 on baseline BMI), baseline SV50, Q50, or HR50 (for training responses only), and up to a third-degree polynomial in age, separately within race-by-sex-by-generation subgroups. Only significant terms (5% level) were retained (i.e., the model did not need to be saturated). The residuals from this regression (or the raw score if no BSA/BMI or age terms were significant) were then standardized to zero mean and unit variance within each subgroup and constituted the phenotype for the present study.

Molecular studies. The original genome-wide linkage scan was based on 509 markers (21), and chromosome 2 was covered with 37 markers (average density, one marker per 7 Mb). For the chromosome 2q31.1 dense mapping, additional microsatellites were selected from the National Center for Biotechnology Information (NCBI) UniSTS database (**http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unists**). The uniqueness of each primer pair was confirmed by performing a BLAST search of the STS sequence against the Human Genome database (**http://www.ncbi.nlm.nih.gov/BLAST**). PCR conditions and genotyping methods have been fully outlined previously (8). Automatic DNA sequencers from LI-COR (Lincoln, NE) were used to detect the PCR products, and genotypes were scored semi-automatically using the software SAGA. Incompatibilities of Mendelian inheritance were checked, and markers showing incompatibilities were re-genotyped completely. The map locations for chromosome 2 microsatellites were taken from the NCBI sequence map (build 30). Single nucleotide polymorphisms (SNPs) were selected from the NCBI SNP database (dbSNP; **http://www.ncbi.nlm.nih.gov/SNP**). The locations of the SNPs in the results section are derived from the complete *TTN* gene sequence (GenBank entry AJ277892).

The SNPs were genotyped using either a primer extension method or the TaqMan assay. For primer extension, the region containing the SNP is first amplified by PCR using flanking primers. An infrared tag-labeled primer is designed so that its 3′ end is located just upstream of the polymorphic site. The initial PCR product is the template for a primer extension reaction performed in the presence of three deoxynucleotides (e.g., dATP, dCTP, and dGTP) and the dideoxynucleotide of the fourth base (e.g., ddTTP). The ddNTP terminator corresponds to one of the alleles of the SNP site. Resulting products of the primer extension reaction are of different lengths for each allele present: a shorter fragment corresponding to the ddNTP terminator base used in the reaction and a longer fragment for the other allele. The primer extension products are detected on 11% polyacrylamide gels run on DNA4200 automated DNA sequencers (LI-COR). SAGA software was then used for semi-automated allele calling. For the TaqMan assay, the DNA region containing the SNP is amplified using flanking PCR primers. The reaction mix also includes two allele-specific fluorogenic probes labeled with different fluorescent reporter dyes at the 5′ end [carboxyfluorescein (FAM) and 6-carboxy-*N,N,N′,N′*-tetramethylrhodamine (TAMRA)] and a quencher dye at the 3′ end (the butylhydroquinones BHQ-1 and BHQ-2). In an intact probe, the proximity of the quencher dye reduces the fluorescence signal from the reporter dye. During PCR amplification, the 5′ exonuclease activity of *Taq* DNA polymerase cleaves the probe only when it matches the amplified DNA sequence. The released reporter dye produces an increase in fluorescence signal, which is measured directly in the PCR plates using a FLUOstar Galaxy plate reader (BMG Labtechnologies, Durham, NC). Fluorescence values are then converted to scatter plots to generate four distinct clusters of data points (one cluster for negative controls, one for each of the two homozygous genotypes, and one for the heterozygous genotype). A complete list of the SNPs (including primer and probe sequences) is available at the HERITAGE Family Study web site (**http://www.pbrc.edu/heritage/home.htm**).

Linkage and association analyses. Linkage analyses were performed with the sib-pair linkage procedure (10, 15) as implemented in the SIBPAL program of the SAGE 4.2 Statistical Package (24). Briefly, if there is a linkage between a marker locus and a putative gene influencing the phenotype, then sibs sharing a greater proportion of alleles identical-by-descent (IBD) at the marker locus also will show a greater resemblance in the phenotype. The phenotypic resemblance of the sibs, modeled as the mean-corrected trait cross-product of the sibs' trait values, is linearly regressed on the estimated proportion of alleles that the sib-pair shares IBD at each marker locus. Both single- and multi-point estimates of allele sharing IBD were generated using the GENIBD program of SAGE 4.2.

Transmission/disequilibrium tests (TDT) among the microsatellite markers were performed using variance-components-based orthogonal model as implemented in the QTDT software package (1). The IBD estimates for the QTDT were generated using SIMWALK2 program. Associations between the titin SNPs and SV50, Q50, and HR50 training responses were analyzed using MIXED model in the SAS software package. Non-independence among family members was adjusted for using a "sandwich estimator," which asymptotically yields the same parameter estimates as ordinary least squares or regression methods, but the standard errors and consequently hypothesis tests are adjusted for the dependencies. The method is general, assuming the same degree of

dependency among all members within a family. The pair-wise linkage disequilibrium (LD) between the SNPs was assessed using the r^2 measure, which is defined as $D^2/(p_1p_2q_1q_2)$, where p_1,q_1 and p_2,q_2 are the allele frequencies of SNP1 and SNP2, respectively, and $D = x_{12} - p_1p_2$ [LD coefficient, difference between observed (x_{12}) and predicted (p_1p_2) haplotype frequencies] (18). The haplotype frequencies were estimated using the EH program (28). A graphical summary of the pair-wise LD values was generated using the GOLD software (2).

RESULTS

In the original genome-wide scan, the suggestive linkage for submaximal exercise stroke volume training response (ΔSV50) on chromosome 2q31-q32 was detected with markers *D2S335* and *D2S1391* located about 12 Mb apart (21). Although the evidence for linkage was fairly modest ($P = 0.006$), the presence of the genes encoding titin in the region prompted us to test whether markers in the *TTN* locus would provide further support for the linkage results. In the first phase, we genotyped one new microsatellite marker located in intron 10 of the *TTN* gene (*D2S324*). The marker increased the evidence of linkage from $P = 0.006$ (original scan) to $P = 0.0002$. The finding was confirmed with another marker in the *TTN* gene (*D2S385*; intron 43). Consequently, we added 10 more microsatellites to increase the average marker density to one per 2.3 Mb. The results of the linkage analyses with the complete marker set on chromosome 2 for ΔSV50 are shown in Fig. 1. The peak linkages were detected with markers *D2S148*, *D2S385*, and *D2S324* ($P = 0.0002$ and $P = 0.00002$ in multi- and single-point analyses, respectively).

Markers *D2S324* and *D2S148* showed also the strongest evidence of linkage with submaximal exercise cardiac output training response (ΔQ50), although the signal was not as strong as for ΔSV50 (Fig. 2). Submaximal exercise heart rate training response (ΔHR50) provided no evidence of linkage with the *TTN* locus (data not included in the original scan), but marker *D2S1776* about 10 Mb upstream of the *TTN* region showed promising linkage with heart rate training response ($P = 0.001$; Fig. 2). A TDT was performed using a set of 10 microsatellite markers covering a 20-Mb region around the *TTN* locus. The only marker showing significant evidence of linkage disequilibrium (nominal $P \leq 0.005$, i.e., $P \leq 0.05$ after correcting for the number of markers) with ΔSV50 ($P = 0.004$) and ΔQ50 ($P = 0.005$) was *D2S385*. None of the markers indicated linkage disequilibrium with ΔHR50.

A panel of 44 SNPs covering the *TTN* gene was selected from the NCBI SNP database (dbSNP) for association studies. Heterozygosity of the markers ranged from 0.032 to 0.458. These markers could be reduced to six subgroups based on the level of pair-wise linkage disequilibrium (Fig. 3). The first group consisted of six markers located in the Z-line region of the gene (rs719201, rs1552280, rs2054707, rs2306637, rs2291303, rs1872202; 15–42 kb in Fig. 3). These SNPs were in complete linkage disequilibrium (LD), and the frequency of the rare allele was 0.8%. Despite the low allele frequency, subjects carrying the rare allele ($n = 8$; all heterozygotes) showed lower ΔSV50 values than the common allele homozygotes ($P = 0.055$; Fig. 4*A*).

The second LD subgroup consisted of seven SNPs spanning the I/A junction and A-band regions of the gene (145–270 kb in Fig. 3). A haplotype representing the group was constructed using markers rs2472751 (145,276 nt) and rs2288571 (241,067 nt). The haplotype showed no association with ΔSV50 in the whole cohort. However, there was a significant haplotype-by-generation interaction effect ($P < 0.001$), the rare haplotype being associated with lower phenotypic values in offspring and higher values in parents (Fig. 4*B*). Interestingly, there was no linkage disequilibrium between the Z-line region markers and the A-band haplotype, i.e., all heterozygotes for the Z-band markers were homozygotes for the common A-band haplotype.

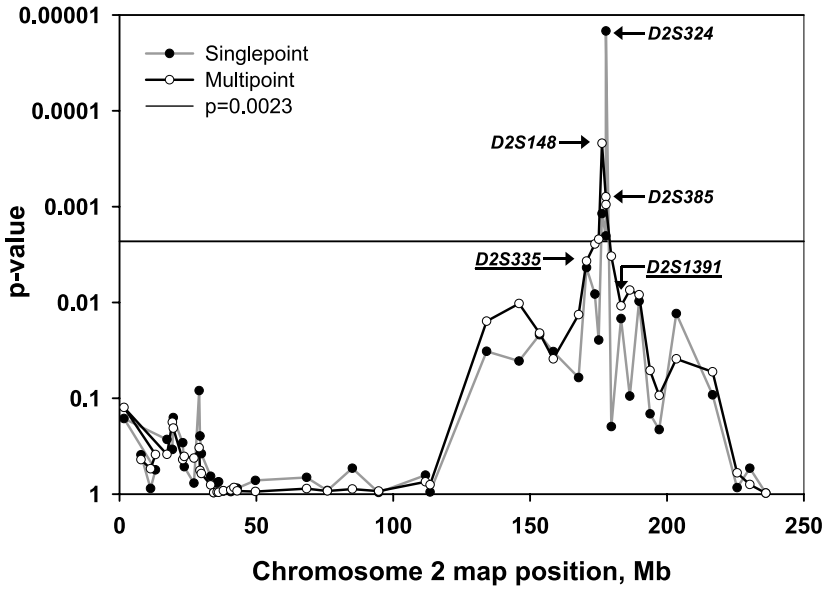

Fig. 1. Single- and multi-point linkage results for the submaximal exercise (50 W) stroke volume (SV50) training response with a dense microsatellite marker set on chromosome 2q31.1 in white HERITAGE subjects. Markers used in the original linkage scan to detect the quantitative trait locus (QTL) are underlined.

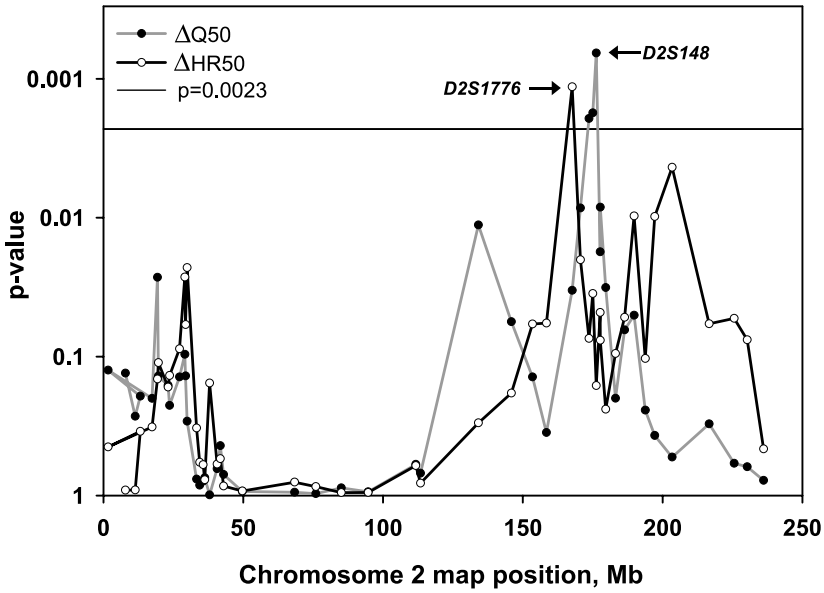

Fig. 2. Multipoint linkage results for Q50 and HR50 training responses on chromosome 2 in white HERITAGE subjects. HR50, heart rate determined 50 W; Q50, cardiac output determined at 50 W.

The remaining four SNP subgroups showed no evidence of association with ΔSV50, ΔQ50, or ΔHR50 in the whole cohort or by generation. One of these subgroups consisted of five markers covering the cardiac-specific N2B exon (~75 kb in Fig. 3).

DISCUSSION

The main finding of the present study is that the original linkage signal for ΔSV50 on chromosome 2q31, which was then considered only suggestive, was enhanced by denser microsatellite mapping. The decision to further investigate this QTL was mainly motivated by the fact that a gene encoding titin is located within the region. Thus, reviewing physiologically motivated candidate genes within a chromosomal region characterized by modest evidence of linkage, using traditional statistical criteria, appears to have led us into an interesting result. Relying on very conservative criteria, especially when marker density is relatively low, may inflate the false-negative rate (22). We therefore

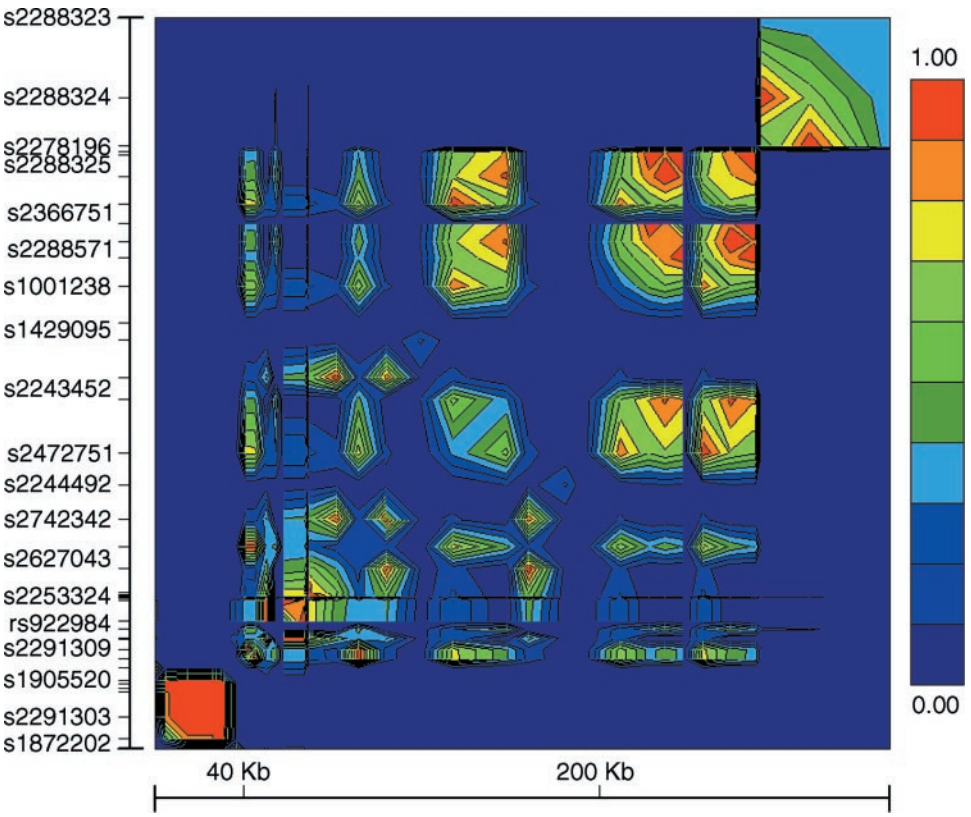

Fig. 3. Summary of the pair-wise linkage disequilibriums (r^2) between the titin (*TTN*) single nucleotide polymorphisms (SNPs). Dark red and dark blue indicate the upper (strong LD) and lower (no LD) extremes of the r^2 distribution, respectively. The *x*-axis indicates the physical distance (from the *TTN* sequence AJ277892) between the SNPs in kilobases, and the *y*-axis gives the position of the SNPs. The graph is generated using the GOLD software package (2). LD, linkage disequilibrium.

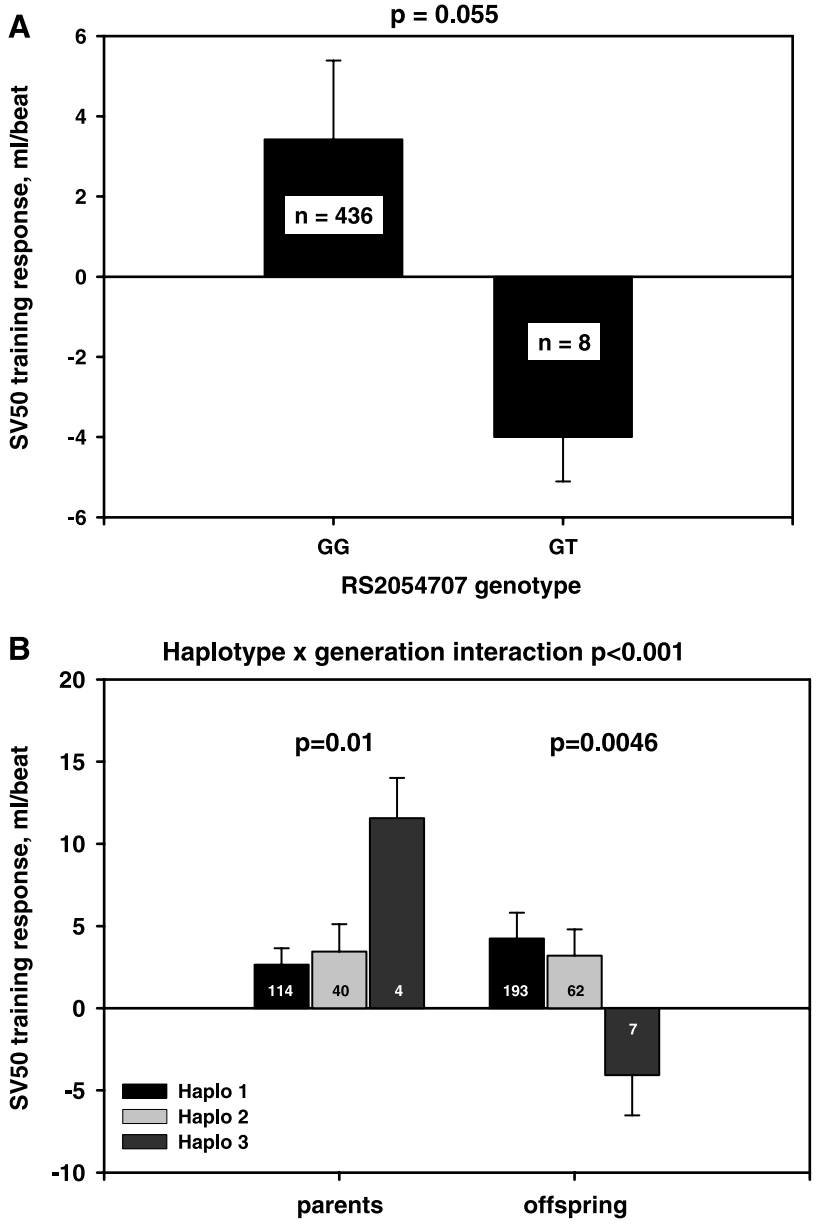

Fig. 4. SV50 training responses according to SNPs characterizing the Z-line region (*A*) and the I/A junction and A-band regions (*B*) of the titin gene. Marker rs2054707 represents six SNPs covering 26.4-kb region that were in complete linkage disequilibrium. Haplotype in *B* summarizes information from seven SNPs covering 125-kb area (from intron 150 to 337). Haplo1 = homozygotes for the common haplotype; Haplo2 = heterozygotes; Haplo3 = homozygotes for the rare haplotype.

recommend that instead of automatically rejecting moderate linkage signals, relevant chromosomal regions should be carefully inspected for potential candidate genes.

The results of the linkage analyses, TDT with microsatellite markers, and association studies with SNPs suggest that titin is a good candidate gene on chromosome 2q31.1 for the SV50 training response in white HERITAGE families. Titin is also a biological candidate for the adaptation of cardiac function to endurance training. It is the third most abundant protein in striated muscle cells and it serves several important roles in the development and function of the myocytes. Titin regulates myofibril formation by providing a scaffold for the assembly of thick and thin filaments (14). It also stabilizes the myosin filaments and maintains structural integrity of the sarcomere. Disruption of titin by mutations or by titin antisense oligonucleotides has been reported to cause impaired sarcomerogenesis and result in thin, poorly contractile muscle cells (19, 29, 34). Titin contains a kinase domain and binding sites for several contractile proteins and intermediate filaments, suggesting a role in intracellular signal transduction pathways. Titin is responsible for the passive forces of a myocyte through its extensible I-band region. The I-band region consists of two main segment types, the PEVK segment and serially linked immunoglobulin (Ig)-like domains. Several splice isoform variants of the I-band segment with different number of Ig domains and varying size of the PEVK segments have been reported. In addition to the common segments, the splice isoforms have different unique sequences encoded by the N2A and N2B exons. The N2A isoform is expressed exclusively in skeletal

muscle, whereas cardiac myocytes express isoforms containing either only N2B sequence or both N2A and N2B elements (N2BA isoform).

The elastic properties of the cardiac titin isoforms differ greatly, with the larger N2BA isoform being less stiff. The relative level of expression of the cardiac titin isoforms have been shown to contribute to differences in cellular stiffness of myocardium across mammalian species and to contribute to the diastolic properties of the heart (7, 33). Furthermore, titin has been suggested to be a main regulator of the Frank-Starling mechanism at the molecular level (11). The elastic properties of titin isoforms provide a reasonable explanation for our findings. It has been suggested that exercise training alters compliance characteristics of the left ventricle in response to acute volume load to the heart during exercise. In sedentary subjects, the Frank-Starling mechanism is the main contributor to stroke volume and cardiac output especially during submaximal exercise, whereas heart rate and myocardial contractility are predominant determinants at maximal exercise (20, 27). Furthermore, elite athletes have an increased capacity to utilize the Frank-Starling mechanism to increase SV during exercise. Against this background, it is possible that some of the interindividual differences in the endurance training-induced changes in SV50 in white HERITAGE subjects could be due to titin-related elastic properties of myocardium.

The association studies with the *TTN* SNPs provided some evidence for associations with ΔSV50, but the evidence is not yet fully conclusive. The SNPs were identified from the public SNP database, and since they were mainly derived from a limited number of chromosomes, they most likely represent only a small proportion of the total DNA sequence variation at the *TTN* locus. Our results suggest that there may be several mutations affecting ΔSV50 in the white HERITAGE families. This would not be surprising considering the numerous functional regions of the titin molecule. Mutation in any of them would potentially change the properties of the molecule and affect the response of cardiac function to exercise training. This scenario is compatible with observations made on cardiomyopathy patients. Three studies have reported seven titin mutations in such patients, all of them located in different regions of the gene (13, 16, 25). Furthermore, a mutation in the cardiac-specific N2B exon of the *TTN* gene results in cardiomyopathy in zebrafish (34). Thus identification of the mutations affecting ΔSV50 in the HERITAGE subjects warrants specific SNP screening by sequencing potentially informative subjects. However, considering the size of the gene (363 exons, ~300 kb) (4) and the number of potential sites affecting the function of the molecule, the sequencing is likely to be a major undertaking.

Although both linkage and association results support the view that DNA sequence variation at the *TTN* locus may contribute to the interindividual differences in ΔSV50, these findings must be viewed against the bigger context. First, the strongest evidence of linkage for the ΔSV50 in the original genome-wide linkage scan was observed on chromosome 10p11. Despite the improved linkage in the present study, the signals for the *TTN* locus were still lower than those for the chromosome 10p11 QTL. These findings suggest that there may be at least two genes/chromosomal regions with moderate contribution to the genetic effect on ΔSV50, which has been estimated to be about 29% of the total variation in the white HERITAGE families (3). Furthermore, even the improved linkage on chromosome 2q31 is still below the threshold proposed for a significant linkage ($P = 2.2 \times 10^{-5}$) in genome-wide scans (17). Thus we cannot completely exclude the possibility of type 1 error. Second, the results for the *TTN* locus were observed only in white subjects, not in the black HERITAGE families. This may be due to the smaller sample size and lower statistical power for black families, or it may reflect a population-specific effect possibly due to interactions with other genes and environmental factors. Third, the linkage with the *TTN* locus was observed only for the SV50 training response, whereas SV50 measured in the sedentary state showed no linkage on chromosome 2. This suggests that titin might modify the cardiac adaptation when challenged by the regular physical stress of exercise, whereas its role in stroke volume regulation in a homogeneous sedentary environment may be negligible. Finally, the CO_2 rebreathing technique used to assess cardiac output and stroke volume in the present study should be considered as an indirect method compared with techniques such as nuclear and magnetic resonance imaging, which provide more direct assessment of myocardial function. Thus there is a possibility that our SV50 and Q50 phenotypes may be affected by some noncardiac factors, such as changes in plasma volume. However, the good reproducibility of the assessments and the fact that both pre- and posttraining phenotypes represented a mean of four individual measurements strongly suggest that the level of random phenotypic variation of SV50 and Q50 is as low as possible.

In conclusion, the present data indicate that the previously reported moderate evidence of linkage for submaximal exercise stroke volume training response on chromosome 2q31.1 was enhanced by a denser marker map. Titin is biologically and statistically a good candidate gene within the region.

DISCLOSURES

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